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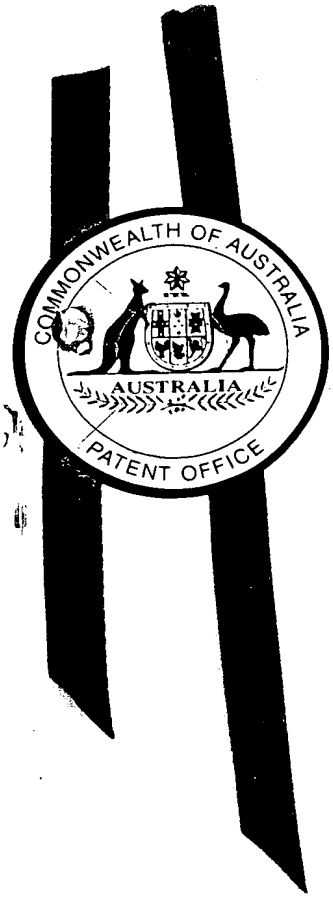

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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s): ST. VINCENT'S INSTITUTE OF MEDICAL RESEARCH

Invention Title: REGULATION OF ENZYME ACTIVITY

The invention is described in the following statement:

REGULATION OF ENZYME ACTIVITY

This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular regulation of activity of endothelial nitric oxide synthase. We have found that the activity of endothelial nitric oxide synthase is regulated by its phosphorylation by several protein kinases including protein kinase C and the AMP-activated protein kinase.

BACKGROUND OF THE INVENTION

Nitric oxide (NO) has recently been recognised as an important mediator of a very wide variety of cellular functions, and is present in most if not all mammalian cells. It is implicated in a range of disorders, hypertension, hypocholesterolaemia, diabetes, heart failure, aging, inflammation, and the effects of cigarette smoking, and is especially important in vascular biology. NO is synthesised from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS have been identified: neuronal NOS (nNOS), which is found in neuronal tissues and skeletal muscle; inducible NOS (iNOS), found in a very wide variety of mammalian tissues including activated macrophages, cardiac myocytes, glial cells and vascular smooth muscle cells; and endothelial NOS (eNOS), found in vascular endothelium, cardiac myocytes and blood platelets.

These three isoforms have an amino acid sequence identity of approximately 55%, with strong sequence conservation in regions involved in catalysis. For all three isoforms, the mechanism of NO synthesis involves binding of the ubiquitous calcium regulatory protein calmodulin (CaM) to the enzyme. However, the conditions under which CaM is bound appear to be different for iNOS, at least insofar as calcium concentration is concerned. These three NOS enzymes have been intensively studied, and the field has been recently reviewed; see for example

Michel and Feron (1997); Harrison (1997); and Mayer and Hellens (1997). Although it was known from earlier studies that eNOS could be multiply phosphorylated, the mechanism of these phosphorylation events, including the enzyme responsible for phosphorylation, and the role of phosphorylation in modulation of eNOS function was not known.

AMP-activated protein kinase (AMPK) is a metabolic stress-sensing protein kinase that is known to play an important role in the regulation of acetyl-CoA carboxylase, leading to the acceleration of fatty acid oxidation during vigorous exercise or ischaemia. AMPK is well known as a regulator of lipid metabolism, and in particular is known to have a role in cholesterol synthesis, reviewed in Hardie and Carling (1997).

AMPK has mainly been studied in the liver, heart and skeletal muscle. AMPK has been purified, and the genes encoding the enzyme subunits have has been cloned (see International Patent Applications No. PCT/GB94/01093 and No. PCT/US97/00270).

The mammalian AMPK (Mitchelhill *et al*, 1994) is related to the *Saccharomyces cereviseae* SNF1 protein kinase, which is required for the expression of glucose-repressed genes during nutritional stress, permitting growth on alternative carbon sources (Celenza and Carlson, 1986); both the mammalian and yeast kinases are activated by upstream kinases (Hardie and Carling, 1997). The AMPK is involved in metabolic stress responses through phosphorylation and concomitant inhibition of acetyl-CoA carboxylase and HMG-CoA reductase (Hardie and Carling, 1997). Multiple AMPK isoforms occur, comprising heterotrimers of $\alpha 1$ or $\alpha 2$ catalytic subunits (63 kDa) (Stapleton *et al*, 1996; Stapleton *et al*, 1997a), together with β (40 kDa) and γ (38 kDa) non-catalytic subunits (Mitchelhill *et al*, 1994; Carling *et al*, 1994; Stapleton *et al*, 1994), related to the yeast sip1p and snf4p respectively.

The gene encoding the α2 sub-unit of AMPK has been localised to chromosome 1 Beri *et al* (1994), while the gene for the α1 sub-unit is on chromosome 5, and those for the β1 and γ1 sub-units are both on chromosome 12; the gene encoding the γ2 sub-unit is localised on chromosome 7 (Stapleton *et al*, 1997).

One of the genes encoding eNOS is on chromosome 7, close to the gene for the γ2 sub-unit of AMPK. Another gene encoding nNOS is found on chromosome 12. (The human gene map SEE http://www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96/tsrch?QTEXT=nitric+oxide+synthase)

Recent work has shown that the AMPK in cardiac and skeletal muscle is activated by vigorous exercise or ischaemic stress (Winder and Hardie, 1996; Vavvas, *et al*, 1997; Kudo *et al*, 1995). This led us to investigate the localization of the AMPK isoforms in these tissues. The AMPK-α2 isoform is present in capillary endothelial cells in cardiac and skeletal muscle and the AMPK-α1 isoform occurs in cardiac myocytes and vessels (results not shown). The presence of AMPK in endothelial cells prompted us to test bacterially expressed eNOS as a substrate, and we found that it is readily phosphorylated by either AMPK-α1 or AMPK-α2.

We have now surprisingly found that the AMP-activated protein kinase phosphorylates and regulates endothelial NO synthase. We find that the AMPK phosphorylates eNOS at two sites. In the presence of calcium and calmodulin, Ser-1177 is phosphorylated in the COOH-terminal tail of the enzyme, causing activation of eNOS by shifting the calmodulin-dose dependence. In the absence of added calcium and calmodium, phosphorylation also occurs at Thr-495 in the eNOS calmodulin-binding sequence, and inhibits the enzyme. Ischaemia of the heart causes activation of the AMPK and of eNOS, mimicking the effects of phosphorylation at Ser-1177. Phosphopeptide specific antibodies to phosphorylated Ser-1177 were used to

confirm that this site was phosphorylated during ischaemia. Phosphorylation of eNOS at Thr-495 by protein kinase C occurs in endothelial cells that have been serum starved and incubated in calcium free media in the presence of phorbol ester. Our results are of special interest because they define a link between metabolic stress that reduces ATP and increases AMP and signalling through eNOS to control nutrient supply via arterial vasodilation, as well as reducing myocardial contraction, thus coupling the metabolic status of endothelial cells and myocytes with the vascular supply and mechanical activity. Our results provide a new insight into the post-translational regulation of eNOS that is of particular significance for the cardiovascular and skeletal muscle field.

SUMMARY OF THE INVENTION

According to a first aspect, the invention provides a method of identifying modulators of AMPK-mediated activation of eNOS, comprising the step of testing putative modulators for their ability to increase or decrease phosphorylation of eNOS depending on the calmodulin and calcium ion concentrations.

Preferably the specific phosphorylation of Ser-1177 is assessed in the presence of calcium and calmodulin.

In an alternative aspect, the invention provides a method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions. Preferably specific phosphorylation of threonine 495 is assessed.

Compounds able to increase phosphorylation of Ser-1177 or decrease phosphorylation of Thr-495 are referred to herein as activators, and compounds able to decrease phosphorylation of Ser-1177 or increase phosphorylation of Thr-495 are referred to as inhibitors.

In both aspects of the invention, one or more of the following activities may optionally be additionally assessed for each putative activator or inhibitor identified by the method of the invention:

1. Effect on smooth muscle contraction;
2. Effect on inotropic activity of the heart;
3. Effect on chronotropic activity of the hear.

It is expected that because the phosphorylation site equivalent to Thr-495 in the eNOS calmodulin-binding site is absent from the neuronal form of NOS, inhibitors and activators identified by the method of the invention will have at least some degree of tissue specificity.

Compounds that activate the AMP-activated protein kinase are expected to be useful in ischaemic heart disease both by promoting glucose and fatty acids metabolism as well as by increasing NOS activity to improve nutrient and oxygen supply to the myocytes and reduce mechanical activity. These compounds would also have utility in pulmonary hypertension and in obstructive airways disease.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows immunofluorescence localization of AMPK-α2 in the heart and in the tibialis anterior muscle.

Panel A shows a negative control section of rat heart stained with control rabbit IgG and control mouse IgG, together with anti-rabbit-FITC and anti-mouse-Texas Red.

Panel B shows a section of rat heart stained with affinity-purified rabbit polyclonal antibody against AMPK-α2 (490-514) and anti-rabbit-FITC.

Panel C shows the same section as Panel B, stained with a monoclonal antibody against rat endothelium recA-1 and anti-mouse-Texas Red.

Panel D shows the overlay of Panels B and C. Colocalization can be seen by the coincidence of staining. The arrows highlight specific endothelial cells that are stained by both antibodies.

Panel E shows a negative control section of rat tibialis anterior muscle stained with control rabbit IgG and control mouse IgG, together with anti-rabbit-FITC and anti-mouse-Texas Red.

Panel F shows a section stained with affinity-purified rabbit polyclonal antibody against AMPK-α2 (490-514) and anti-rabbit-FITC.

Panel G shows the same section as in Panel B, stained with a monoclonal antibody against rat endothelium recA-1 and anti-mouse-Texas Red.

Panel H shows the overlay of Panels E and F. Colocalization can be seen by the coincidence of staining.

Figure 2 illustrates phosphorylation of recombinant eNOS by AMPK.

Top panel: eNOS was incubated with rat liver AMPK-α1 and [γ-^{32}P] ATP.

Lane 1: Coomassie-stained SDS-PAGE;

Lane 2: Autoradiograph.

Lower panel: ^{32}P-tryptic phosphopeptide map of eNOS.

Figure 3 shows the effect of phosphorylation of eNOS by the AMPK with or without added Ca^{2+}-CaM. Rat heart eNOS purified by 2',5'-ADP-Sepharose affinity chromatography was phosphorylated by AMPK in the presence of 0.8 μM CaM/3.2 μM Ca^{2+} (closed circles), in the absence of Ca^{2+}-CaM (closed triangles) and without AMPK (open squares). After phosphorylation, the samples were diluted, eNOS activity was measured. The lower panels show phosphopeptide maps for rat heart eNOS phosphorylated in the presence and absence of added Ca^{2+}-CaM.

Figure 4 shows the effect of ischaemia on the activities of AMPK-α1, AMPK-α2 and eNOS.

Panel A shows the results of immunoprecipitation using antibody specific for AMPK-α1 and AMPK-α2, assayed using the SAMS peptide substrate. Results shown are mean ± SEM for n=5.

Panel B shows eNOS activity measured at 500 nM CaM.

Panel C shows eNOS activities with full CaM-dose responses for a representative experiment. Ischaemia time points: 0 min (open squares), 1 min (closed diamonds), 10 min (closed circles) and 20 min (open triangles). The results of 4 replicates were the same, except that in one case the 20 min ischaemia eNOS CaM-dependence remained the same as for 10 min.

Figure 5 shows a comparison of NOS phosphorylation site sequences for eNOS, nNOS and iNOS, in a schematic model of NOS. Sequences from the CaM-binding region (around the Thr-495 phosphorylation site in eNOS) and for the COOH-terminal tail (around the Ser-1177 phosphorylation site in eNOS) are shown.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following non-limiting examples and to the figures.

We have surprisingly found that in the presence of Ca^{2+}-calmodulin (CaM) eNOS is phosphorylated by AMPK at Ser-1177, resulting in activation, whereas phosphorylation of eNOS in the absence of Ca^{2+} occurs predominantly at Thr-495, a site in the CaM-binding sequence, resulting in inhibition. It had previously been considered that phosphorylation was solely inhibitory. We have also found that ischaemia of the heart leads to rapid activation of both isoforms of the metabolic stress-sensing enzyme AMPK and eNOS. These data suggest that the AMPK may operate an "inside-out" signalling pathway that leads to arterial vasodilation and reduced myocardial contraction, so

coupling the metabolic status of endothelial cells and myocytes with the vascular supply and mechanical activity.

Example 1 Immunofluorescence Localisation of AMPK-α2 in Heart and Skeletal Muscle

Confocal immunofluorescence microscopy using affinity-purified rabbit polyclonal antibody directed against AMPK-α2 (antibody 490-414); (Stapleton *et al*, 1996) and staining with fluorescence-labelled anti-rabbit antibody showed that the α2 isoform is found predominantly in capillary endothelial cells in both cardiac muscle and skeletal muscle, while cardiac myocytes and blood vessels showed intense but diffuse staining for the α1 isoform of AMPK. In skeletal muscle, the α2 isoform was found in endothelial cells of capillaries, and in fast-twitch muscle fibres, whereas the α1 isoform was found in Type I aerobic fibres. Localisation of AMPK-α2 in capillary endothelial cells in both cardiac and skeletal muscle is illustrated in Figure 1.

Example 2 AMPK Phosphorylates Recombinant eNOS

Bacterially expressed eNOS, coexpressed with CaM by the method of Rodriguez-Crespo *et al* (1996), was phosphorylated by either AMPK-α1, as shown in Figure 2 top panel, or AMPK-α2. Recombinant eNOS phosphorylation by immunoprecipitated AMPK-α2 (Stapleton *et al*, 1996) was detected. Since we have been unable to purify high specific activity AMPK-α2, no further characterization of eNOS regulation or the sites of phosphorylation by the α2 isoform was undertaken. Analysis of the phosphorylation sites in eNOS following tryptic digestion revealed four phosphopeptides generated from three separate sites (Figure 2 bottom panel, A, A', B, C). Identification of phosphorylation sites by mass spectrometry and Edman sequencing, using the method described by Mitchelhill *et al*, 1997a, revealed that Ser-1177 was the most prominent phosphorylation site, as shown in Figure 2 bottom panel, A,

A', and that its phosphorylation was dependent on the presence of Ca^{2+}-CaM.

Phosphopeptide isolation from in-gel tryptic digests was carried out as described by Mitchelhill *et al*, 1997a. Greater than 98% of the radioactivity was recovered from the gel. Peptides isolated and characterized by mass spectrometry and Edman sequencing were set out in Table 1.

Table 1

Phosphopeptides Isolated from In-Gel tryptic Digests

Observed Mass	Phosphopeptide	Sequence	Calculated Mass
1440.0	B	KKTFKEVANAVK	1361.1(*1441.7)
1174.1	A	TQXFSLQER	1094.5(*1174.5)
1445.6	A'	IRTQXFSLQER	1363.7(*1443.7)
1176.7	C	pcLGSLVFPR	1095.6(*1175.6)

where: "pc" denotes pyridylethyl cysteine.

* denotes calculated mass of mono-phosphorylated peptide.

The location of the phosphorylation site in peptide A, TQXFSLQER, was identified by ^{32}P-phosphate release sequencing (Mitchelhill *et al*, 1997a). eNOS phosphorylated by the AMPK-α1 was no longer recognized by the antibody to the eNOS COOH-terminal tail; nor was it elute from the ADP-Sepharose affinity column by 100 mM NADPH. These properties prevented the direct confirmation of Ser-1177 phosphorylation *in situ*. This is illustrated in Venema *et al*, 1996.

A second site, Thr-495, was phosphorylated in the absence of Ca^{2+}-CaM or when EGTA was present. This is illustrated in Figure 2 bottom panel, B. This residue is located in the CaM-binding sequence,

TRKKT^{495}FKEVANAVKISASLM,

between the oxidase and reductase domains of eNOS (Venema *et al*, 1996). Ser-101 in the N-terminal region of eNOS was identified as a minor site of phosphorylation (Figure 2 bottom panel, C).

Synthetic peptides containing Thr-495 or Ser-1177 were readily phosphorylated by AMPK, with similar kinetic values to the SAMS peptide substrate. The peptide containing Thr-495, GTGITRKKTFKEVANAVK, was phosphorylated with a Km of 39 ± 10 μM and a Vmax of 6.7 ± 0.6 μmol/min/mg, whereas the peptide containing Ser-1177, RIRTQSFSLQERQLRG was phosphorylated with a Km of 54 ± 6 μM and a Vmax of 5.8 ± 0.3 μmol/min/mg. These are comparable to results obtained using the well-characterized SAMS peptide substrate, which has a Km 33 ± 3 μM and a Vmax of 8.1 ± 1.5 μmol/min/mg (Michell *et al*, 1996). The *in vitro* phosphorylation of the peptides confirms the identification sites of phosphorylation.

Example 3 Effect of Ca^{2+}-CaM on Phosphorylation of eNOS by AMPK

The eNOS activity was determined by measuring L-[^{3}H]-citrulline production, using the method of Balligand *et al*, 1995. The recombinant eNOS was coexpressed with

CaM, as described by Rodriguez-Crespo and Ortiz de Montellano, 1996. Partially-purified rat heart eNOS contained some Ca^{2+}-CaM. In the absence of added EGTA, CaM dependence was observed at 0-100 nM added CaM. In order to investigate the changes in NOS activity with phosphorylation in the absence and presence of Ca^{2+}-CaM, EGTA buffering was used to achieve CaM dose response curves in the range 0-1 µM. Routinely, 7-15 µM EGTA was added to make eNOS activity dependent upon added CaM. Where Ca^{2+}-CaM was used in the phosphorylation reaction prior to eNOS assay, the samples were either diluted so that the extra Ca^{2+}-CaM was negligible, or the indicated concentrations represent total final concentrations of added Ca^{2+}-CaM.

Cardiac eNOS was partially purified as follows: 20 rat hearts were homogenized in 80 ml of ice-cold buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na Pyrophosphate, 10 µg/ml Trypsin inhibitor, 2 µg/ml Aprotinin, 1 mM Benzamidine, 1 mM PMSF, 10% Glycerol, 1% Triton-X-100]. The homogenate was put on ice for 30 min and centrifuged at 16,000 x g for 30 min. The supernatant was incubated with 2 ml of 2',5'-ADP-Sepharose (Bredt and Snyder, 1990). After one hour incubation, the suspension was poured into a fritted column, which was washed with 20 ml of buffer A and 20 ml of buffer A with 0.5 M NaCl, and then with 20 ml of buffer B [50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% Glycerol, 0.1% Triton-X-100]. eNOS was eluted with buffer B containing 2 mM NADPH, then subjected to centrifugal filtration (ULTRAFREE-MC MILLIPORE) to remove NADPH. Immunoblotting was used for selective detection of eNOS rather than nNOS.

Phosphorylation of eNOS by AMPK in the presence of Ca^{2+}-CaM resulted in activation, but CaM-dependence was retained, as shown in Figure 3 top panel. Activation shifted the dose response curve for CaM to the left. Phosphopeptide mapping revealed that activation of eNOS was

correlated with phosphorylation of Ser-1177 but not of Thr-495, as shown in Figure 3 lower panel. Phosphorylation without added Ca^{2+}-CaM enhanced Thr-495 phosphorylation, suppressed Ser-1177 phosphorylation, and inhibited eNOS activity (Figure 3 top panel). The inhibition of eNOS activity by Thr-495 phosphorylation is consistent with earlier reports that phosphorylation of synthetic peptides corresponding to this region by protein kinase C inhibits CaM-binding (Matsubara *et al*, 1996). Similar results have been reported for nNOS (Loche *et al*, 1997).

Example 4 Effect of Ischaemia on Activities of AMPK-α1, AMPK-α2 and eNOS

Langendorf preparations of isolated perfused rat heart were subjected to ischaemia according to the method of Kudo *et al* (1995). AMPK-α1 and AMPK-α2 isoforms were immunoprecipitated using α2 (490-516) or α1 (231-251) antibodies, and assayed using the SAMS peptide substrate (Michell *et al*, 1996; Hardie and Carling, 1997). eNOS activity was measured as described in Example 3. The results are shown in Figure 4. Both α1 and α2 isoforms, are activated, as shown in Figure 4A, indicating that AMPK is activated in both capillary endothelial cells, which have predominantly the α2 isoform, and in cardiac myocytes, which have predominantly the α1 isoform. AMPK activation during ischaemia is also accompanied by eNOS activation and changes in the CaM dependence, as shown in Figures 4B and 4C, mimicking the effect of eNOS phosphorylation by AMPK *in vitro*, as shown in Figure 3.

Polyclonal antibodies were raised against synthetic phosphopeptides based on the eNOS sequence: RIRTQSpFSLQER and GITRKKTpFKEVANCV. Rabbits were immunized with phosphopeptides coupled to keyhole limpet haemocyanin and then emulsified in Fruend's complete adjuvant, using conventional methods. The antibodies were purified using the corresponding phosphopeptide affinity columns after thorough preclearing with dephosphopeptide affinity

columns. The specificity of the purified antibodies was confirmed using both EIA and immunoblotting, confirming that they did not recognize recombinant dephospho-eNOS.

Using the anti-phosphopeptide antibodies to Ser-1177 and Thr-495 phosphorylation sites we observed that phosphorylation of Ser-1177 was increased approximately 3-fold with ischaemia, but that there was no detectable change in the Thr-495 phosphorylation under these conditions. Heart muscle contains eNOS in both capillary endothelial cells and cardiac myocytes (Balligand *et al*, 1995), with low levels of the nNOS μ isoform (Silvagno *et al*, 1996).

The sequences of the three types of NOS are compared in Figure 5, which shows the CaM-binding region and the C-terminal tail. In nNOS Ser-1417 corresponds to eNOS Ser-1177, whereas iNOS is truncated, and has a Glu in this region. Both iNOS and nNOS lack a phosphorylatable residue equivalent to Thr-495.

Example 5 Effect of Stimulation of Protein Kinase C on eNOS Phosphorylation

Bovine aortic endothelial cells cultured in 0.1% foetal calf serum for 20 hours (serum starved) were subjected to treatment with the protein kinase C activator 0.1 μM phorbol-12-myristate-13-acetate (PMA) for 30 min. PMA treatment increased the phosphorylation of eNOS at Thr-495 and decreased the phosphorylation at Ser-1177, as measured using anti-phosphopeptide specific antibodies. The antibodies used were the same as those described in Example 4. The response was most marked when the cells were incubated in medium lacking added calcium.

DISCUSSION

Activation of eNOS by phosphorylation of its COOH-terminal tail gives new insight into eNOS autoinhibition. The increased activity and shift in the CaM-dose dependence with phosphorylation at Ser-1177

suggest that in eNOS, and perhaps nNOS, the COOH-terminal tails act as partial autoregulatory sequences analogous to those in the CaM-dependent protein kinases (Kemp and Pearson, 1991; Kobe *et al*, 1996).

The COOH-terminal tail of eNOS is only fully accessible to the AMPK when Ca^{2+}-CaM is bound, consistent with this region being buried in the absence of Cam. As can be seen from Figure 5, there is a high level of similarity between eNOS and nNOS in their COOH-terminal tails, whereas iNOS is distinct. It is known that the iNOS CaM-binding, which is characterised by a low Ca^{2+}-dependence, requires both the canonical CaM-binding sequence and distal residues in the COOH-terminus that cannot be satisfied by nNOS chimeras (Ruan *et al*, 1996). Without wishing to be bound by any proposed mechanism, we believe that eNOS and nNOS are autoinhibited by their COOH-terminal tails, requiring a two-stage activation process for full activity with both CaM-binding and phosphorylation in the tail, whereas iNOS requires only CaM binding. Recently, Salerno *et al* (1997) proposed that an insert sequence in the FMN-binding domain may also be important in autoregulation.

Previous studies have shown that eNOS may be phosphorylated both *in vitro* and *in vivo*, but the precise sites of phosphorylation and the function of the phosphorylation events have not hitherto been fully characterized (reviewed in Michel and Feron, 1997). eNOS is the first example of an enzyme activated by AMPK to be identified, and is also unusual because phosphorylation can lead to either activation or inhibition, depending on the availability of Ca^{2+}-CaM. Other enzymes, notably the cyclin-dependent protein kinases, are activated or inhibited by phosphorylation, but this is catalysed by different protein kinases. Protein kinase C phosphorylates Thr-495 in eNOS, demonstrating intersecting regulatory pathways acting on eNOS by phosphorylation of Thr-495 or Ser-1177. It is also possible that persistent activation

of protein kinase C could block eNOS activation by AMPK during metabolic stress.

The regulation of eNOS by AMPK extends the conceptual relationship between the yeast snf1p kinase and the AMPK. Whereas the snf1p kinase modulates the supply of nutrients from the environment by secreting invertase, the mammalian AMPK has been used to integrate metabolic stress signalling with the control of the circulatory system, so that intracellular metabolic stress signals within endothelial cells and myocytes can elicit improved nutrient supply and suppress mechanical activity of the muscle.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

Reference cited herein are listed on the following pages, and are incorporated herein by this reference.

REFERENCES

Balligand, J.L., Kobzik, L., Han, X., Kaye, D.M., Belhassen, L., O'Hara, D.S., Kelly, R.A., Smith, T.W. and Michel, T.
J. Biol. Chem., 1995 270 14582-14586

Beri, R.K. and Marley, A.E.
See, C.G., Sopwith, W.F., Aguan, K., Carling, D., Scott, J., and Carey, F.
Febs Lett, 1994 356 117-121

Bredt, D.S. and Snyder, S.H.
Proc. Natl. Acad. Sci. USA, 1990 87 682-685

Carling, D., Aguan, K., Woods, A., Verhoeven, A.J.M., Beri, R., Brennan, C.H., Sidebottom, C., Davidson, M.D. and Scott, J.
J. Biol. Chem., 1994 269 11442-11448

Celenza, J.L. and Carlson, M.
Science, 1986 233 1175-1180

Hardie, D.G. and Carling, D.
Eur J Biochem., 1997 246 259-273

Kemp, B.E. and Pearson, R.B.
Biochim. Biophys. Acta, 1991 1094 67-76

Kobe, B., Heierhorst, J., Feil, S.C., Parker, M.W., Benian, G.M., Weiss, K.R. and Kemp, B.E.
Embo. J., 1996 15 6810-6821

Kudo, N., Barr, A.J., Barr, R.L., Desai, S., Lopaschuk, G.D.
J. Biol. Chem., 1995 270 17513-17520

Matsubara, M., Titani, K. and Taniguchi, H.
Biochemistry, 1996 35 14651-14658

Michel, T. and Feron, O.
J. Clin. Invest., 1997 100 2146-2152

Michell, B.J., Stapleton, D., Mitchelhill, K.I., House, C.M., Katsis, F., Witters, L.A. and Kemp, B.A.
J. Biol. Chem., 1996 271 28445-28450

Mitchelhill, K.I., Michell, B.J., House, C., Stapleton, D., Dyck, J., Gamble, J., Ullrich, C., Witters, L.A., and Kemp, B.E.
J. Biol. Chem., 1997 272 24475-24479

Mitchelhill, K.I., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L.A. and Kemp, B.E.
J. Biol. Chem., 1994 269 2361-2364

Rodriguez-Crespo, I., Ortiz de Montellano, P.R.
Arch. Biochem. Biophys., 1996 336 151-156

Ruan, J., Xie, Q., Hutchinson, N., Cho, H., Wolfe, G.C. and Nathan, C.
J. Biol. Chem., 1996 271 22679-22686

Salerno, J.C., Harris, D.E., Irizarry, K., Patel, B., Morales, A.J., Smith, S., Martasek, P., Roman, L.J., Masters, B., Jones, C.L., Weissman, B.A., Lane, P. *et al.*
J. Biol. Chem., 1997 272 29769-29777

Silvagno, F., Xia, H. and Bredt, D.S.
J. Biol. Chem., 1996 271 11204-11208

Stapleton, D., Guang, G., Michell, B.J., Widmer, J., Mitchelhill, K.I., Teh, T., House, C.M., Witters, L.A. and Kemp, B.E.
J. Biol. Chem., 1994 269 29343-29346

Stapleton, D., Mitchelhill, K.I., Gao, G., Widmer, J., Michell, B.J., Teh, T., House, C.M., Fernandez, C.S., Cox, T., Witters, L.A. and Kemp, B.E.
J. Biol. Chem., 1996 271 611-614

Stapleton, D.A., Woollatt, E., Mitchelhill, K.I., Nicholl, J.K., Fernandez, C.S., Michell, B.J., Witters, L.A., Power, D.A., Sutherland, G.R. and Kemp, B.E.
FEBS Lett., 1997 409 452-456

Stapleton, D., Woollatt, E., Mitchelhill, K.I., Nicholl, J.K., Fernandez, C.S., Michell, B.J., Witters, L.A., Power, D.A., Sutherland, G.R. and Kemp, B.E.
Febs Lett, 1997 411 452-456

Vavvas, D., Apazidis, A., Saha, A.K., Gamble, J., Patel, A., Kemp, B.E., Witters, L.A. and Ruderman, N.B.
J. Biol. Chem., 1997 272 13255-13261

Venema, R.C., Sayegh, H.S., Kent, J.D., Harrison, D.J.
J. Biol. Chem., 1996 271 6435-6440

Winder, W.W. and Hardie, D.G.
Am. J. Physiol., 1996 270 E299-E304

Zoche, M., Beyermann, M. and Koch, K.W.
Biol. Chem., 1997 378 851-857

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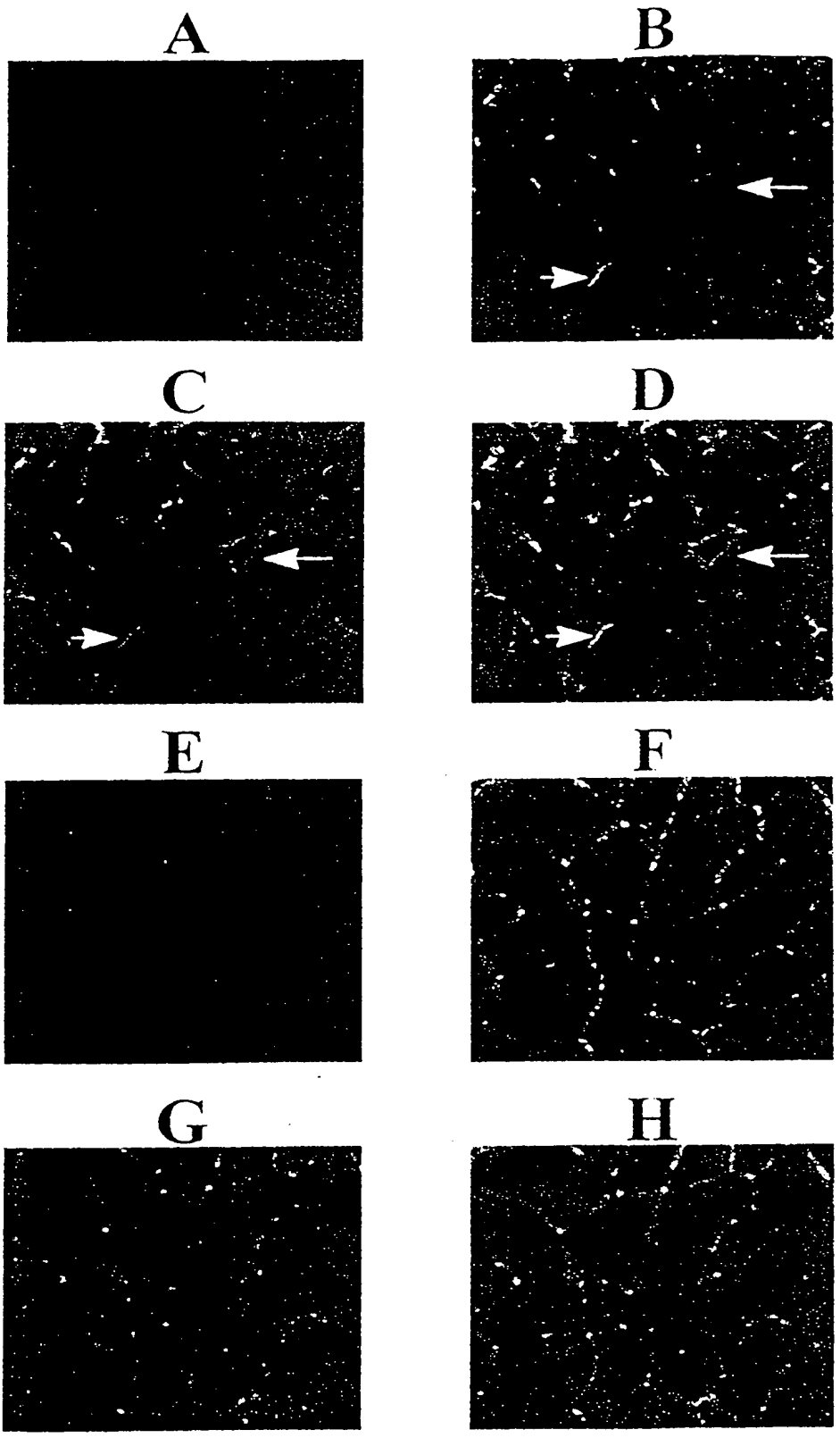
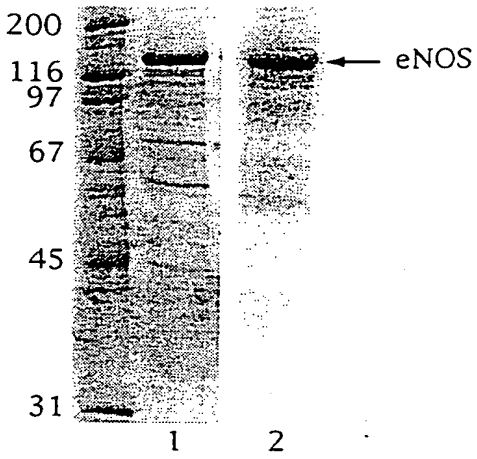


FIGURE 1



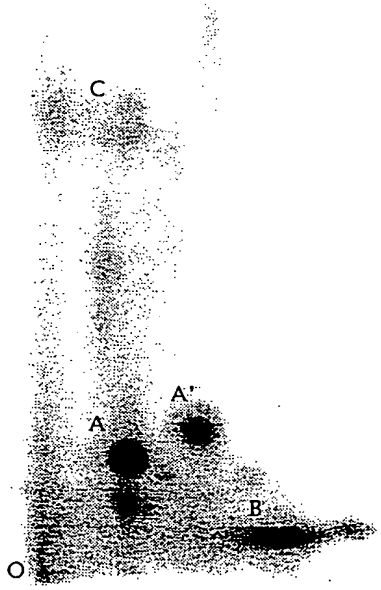
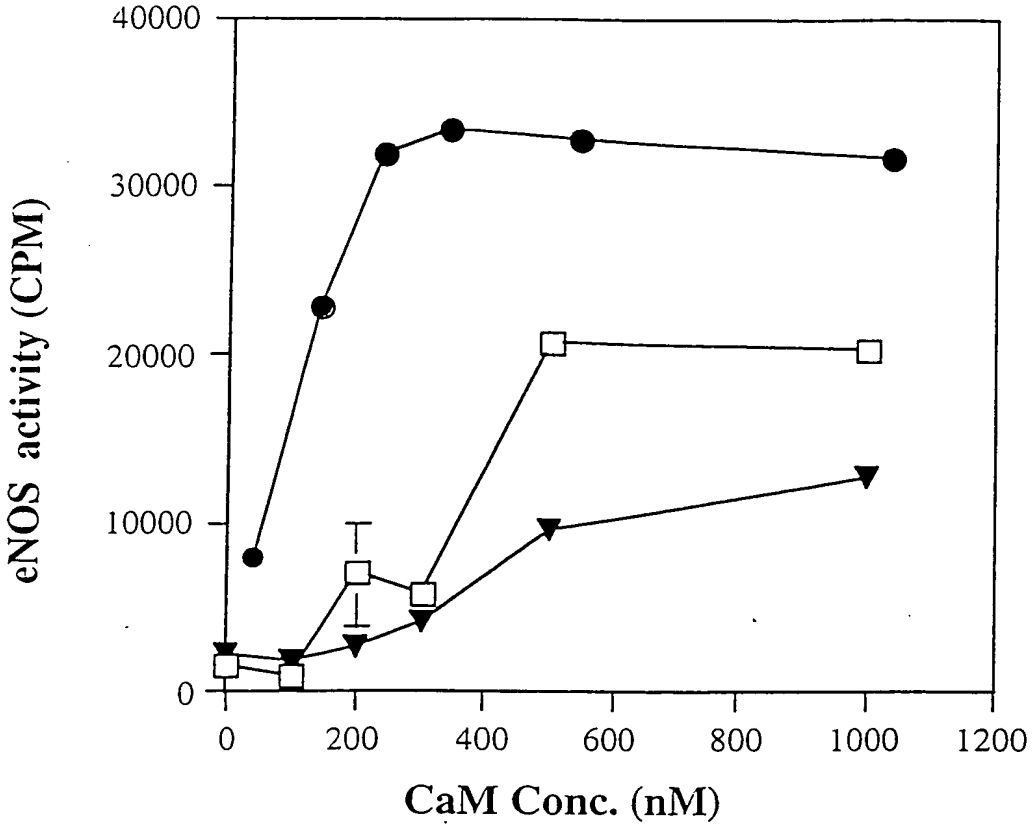
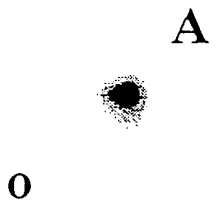


FIGURE 2



eNOS + AMPK
+ Ca^{2+}-CaM



eNOS + AMPK

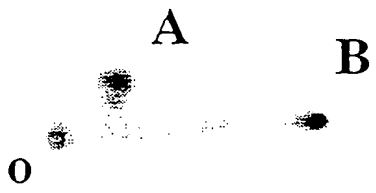


FIGURE 3

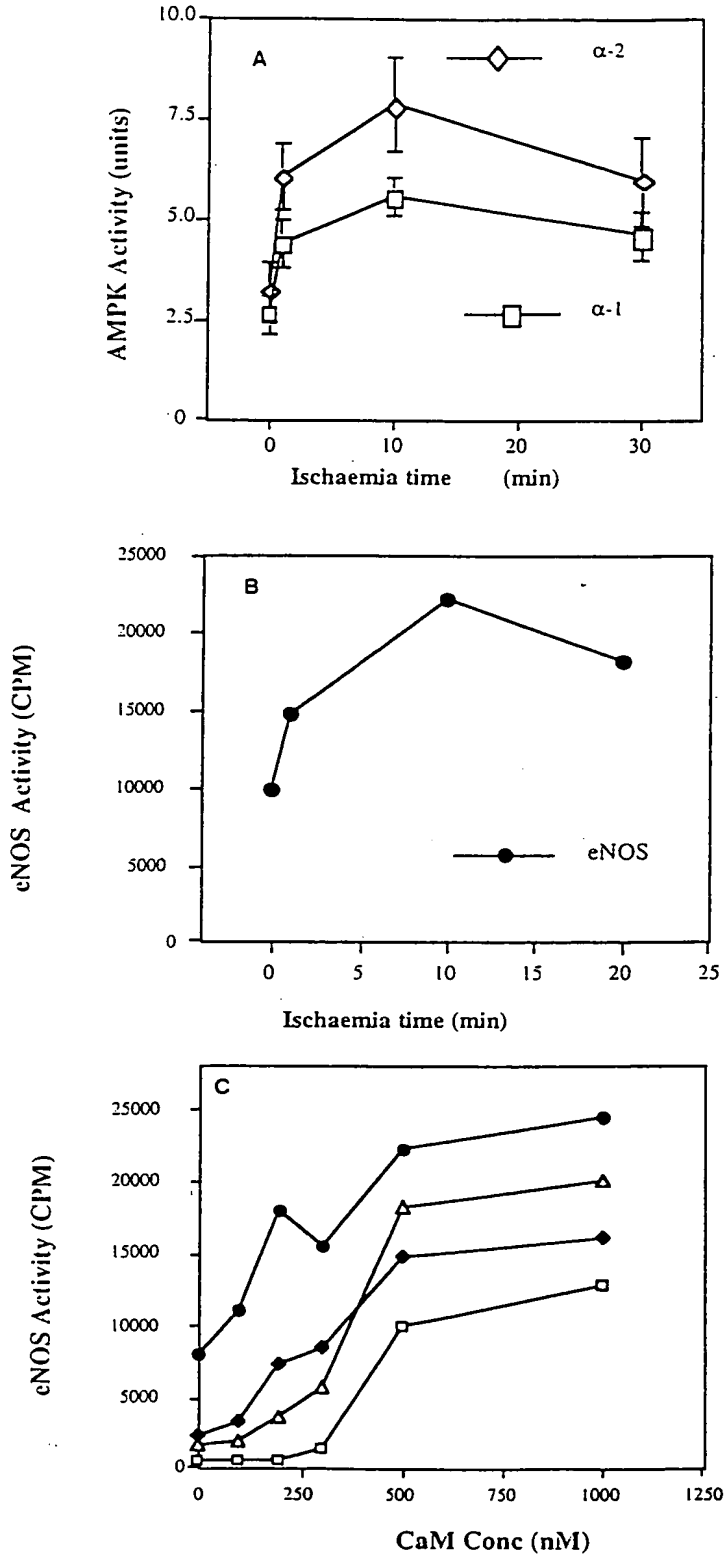

A
α-2
α-1
AMPK Activity (units)
10.0
7.5
5.0
2.5
0
0
10
20
30
Ischaemia time (min)
B
eNOS
eNOS Activity (CPM)
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
Ischaemia time (min)
C
eNOS Activity (CPM)
25000
20000
15000
10000
5000
0
0
250
500
750
1000
1250
CaM Conc (nM)

FIGURE 4

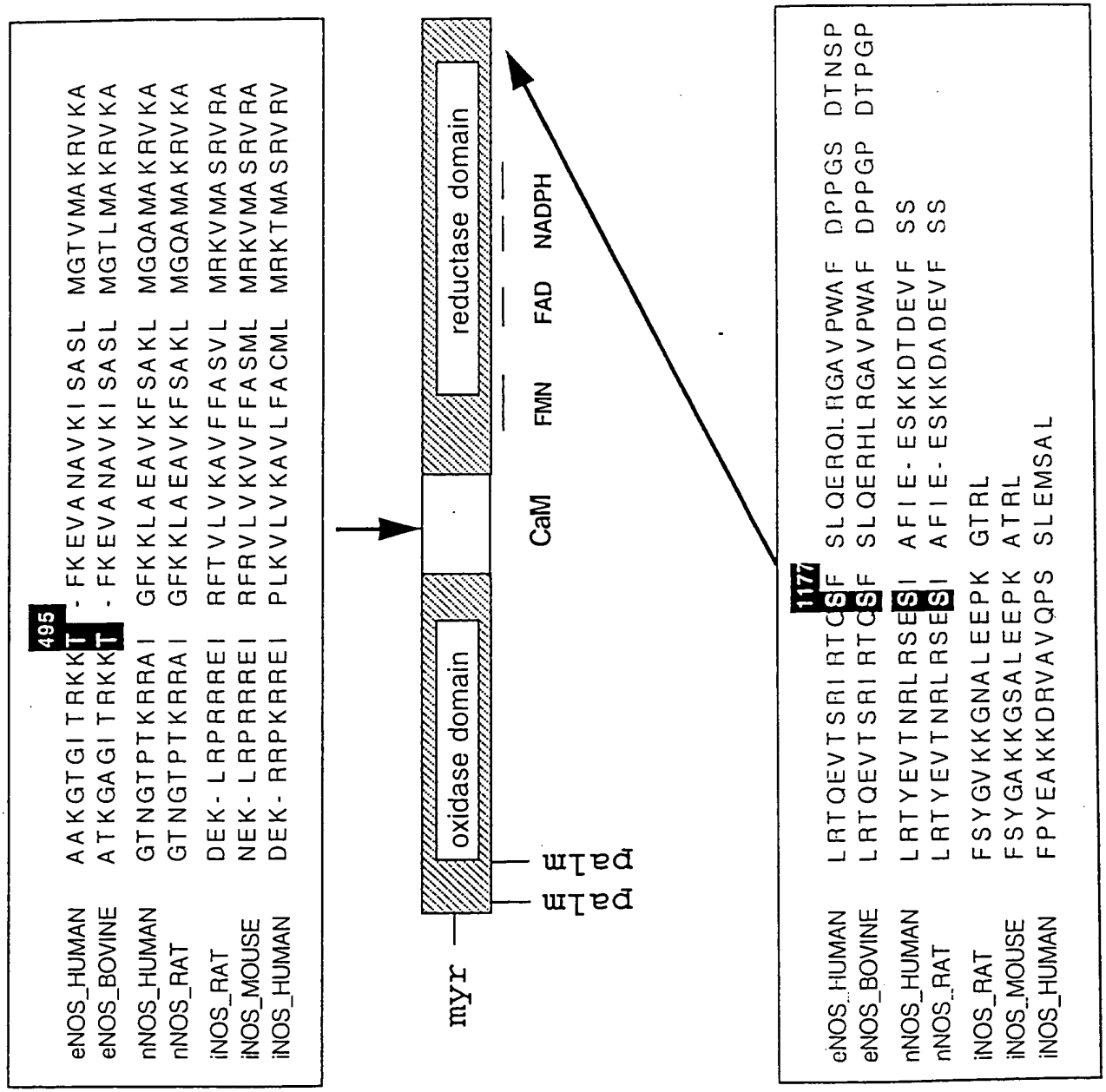

eNOS_HUMAN AAKGTGITRKKT - FKEVANAVKISASL MGTVMAKRVKA
eNOS_BOVINE ATKGAGITRKKT - FKEVANAVKISASL MGTLMAKRVKA
nNOS_HUMAN GTNGTPTKRRAI GFKKLAEAVKFSAKL MGQAMAKRVKA
nNOS_RAT GTNGTPTKRRAI GFKKLAEAVKFSAKL MGQAMAKRVKA
iNOS_RAT DEK-LRPRRREI RFTVLVKAVFFASVL MRKVMASRVRA
iNOS_MOUSE NEK-LRPRRREI RFRVLVKVVFFASML MRKVMASRVRA
iNOS_HUMAN DEK-RRPKRREI PLKVLVKAVLFACML MRKTMASRVRV
495
myr
palm
palm
oxidase domain
reductase domain
CaM
FMN
FAD
NADPH
1177
eNOS_HUMAN LRTQEVTSRIRTGSF SLQERQLRGAVPWAF DPPGS DTNSP
eNOS_BOVINE LRTQEVTSRIRTGSF SLQERHLRGAVPWAF DPPGP DTPGP
nNOS_HUMAN LRTYEVTNRLRSESI AFIE-ESKKDTDEVF SS
nNOS_RAT LRTYEVTNRLRSESI AFIE-ESKKDADEVF SS
iNOS_RAT FSYGVKKGNALEEPK GTRL
iNOS_MOUSE FSYGAKKGSALEEPK ATRL
iNOS_HUMAN FPYEAKKDRVAVQPS SLEMSAL

FIGURE 5